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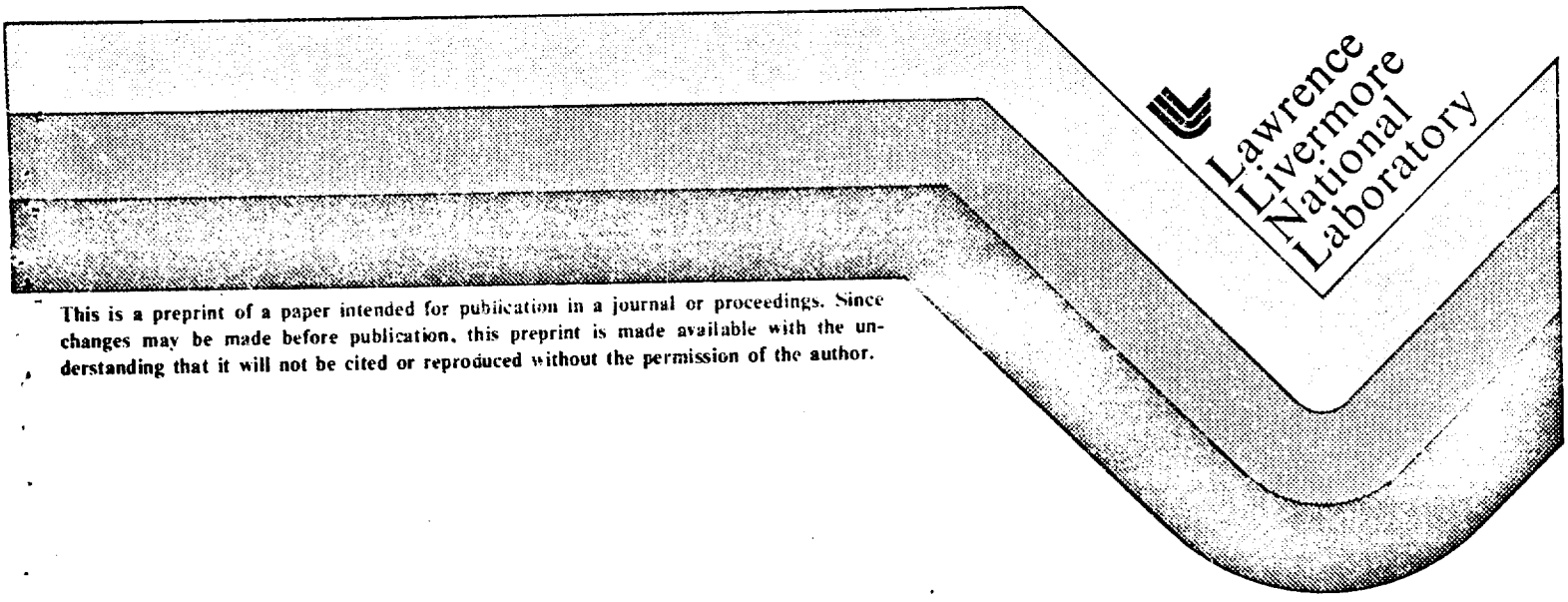

DETECTION OF MUTATED ERYTHROCYTES IN MAN

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INTRODUCTION

We wish to develop methods capable of detecting the results of mutations occuring in single genes in the DNA of somatic cells in people. The great advantage of this approach is that mutant cells, rather than individuals presenting transmitted mutant phenotypes, are identified. The frequency of such cells may represent the integrated rate of mutations occuring in that gene in a particular tissue. Inasmuch as such damage can be taken as representative of the entire genome in every tissue of the body, such a measurement may serve as a monitor of the overall genetic insult, both background and environmentally induced, suffered by that individual. The motivation for such development is both of a practical and basic scientific nature. On the practical side, establishment of such methods would lead to screening procedures to quantify accumulated environmental genotoxic burden to human individuals in the short term. If mutational injury is an initiating mechanism in the development of human cancer, then application of these tests may have important clincal and health monitoring significance. Screening could be used to recognize exposed workers in occupational settings and to identify atypically vulnerable people in the population due to variations in genetic susceptibility and/or lifestyle. On the basic biological side, such methods will measure background mutational frequencies in somatic tissues leading to estimates of mutation rates, correlation with germinal mutation rates and the possible link to carcinogenesis.

To be useful, the assay system must satisfy several genetic and biochemical criteria. Since the frequency of mutant cells is low, the tissue to be sampled must be readily accessible and capable of yielding large numbers of intact single cells. Also, the mutant cells must appear at a measurable

frequency. Here two general strategies have been pursued. The first, for example the detection of thioguanine-resistant lymphocytes,[1] is based on mutations in the X-linked gene for HGPRT which functionally exists in a single copy per cell. Thus a single mutational event, resulting in the lack of functional gene product, will produce a HGPRT "null" cell. For an autosomal gene, such a variant will be extremely rare since a "null" cell could only occur as a result of damage to both copies of the gene in a single cell. A second strategy, and the one employed in our assays, attempts to detect heterozygous normal/mutant cells, i.e., cells containing co-dominately expressed autosomal genes in which one allele is normal and the other is mutant. Such cells should, in the case of neutral mutations, produce both normal and mutant gene products, or in the case of "null" mutations, synthesize protein coded for by only one allele. For accuracy, the mutant cells must not be selected against in vivo and the selection method must be powerful and specific; either clonogenic assay of mutant cells or immunologic detection of mutant gene products. Finally, an independent means must exist to validate that the identified cells result from a structural gene mutation. For example, maintenance of the mutant phenotype under non-restrictive growth conditions or the direct biochemical demonstration of the synthesis of a variant protein.

In this paper, I will described two assay systems we are developing based on high speed cell sorter detection of immunologically identified variant human erythrocytes. Both take advantage of the ability of the flow sorter to rapidly screen large numbers of cells to enumerate rare, presumptively mutant, cells labeled with fluorescent antibodies.

DETECTION OF RARE RED BLOOD CELLS CONTAINING VARIANT HEMOGLOBIN

Nearly 400 variant hemoglobins, the majority characterized by a single amino acid substiution, have been identified in the the human population.(2) Individuals who synthesize any one of these mutant hemoglobins do so as a result of inheriting a mutated hemoglobin gene. The presence of such rare individuals carrying such germinal mutations suggests that these same mutational events may also occur somatically in the hemoglobin genes of the blood-forming stem cells. In normal hemoglobin A individuals, such mutations would give rise to rare circulating red blood cells containing variant hemoglobin in addition to the normal form.

To identify variant hemoglobin-containing cells, Dr. George Stamatoyannapoulos at the University of Washington prepared two antibodies, one specific for hemoglobin S(3) and the other for hemoglobin C.(4) These antibodies recognize and bind tightly to hemoglobin S or C but not to normal hemoglobin A. These two variant hemoglobins differ from the normal hemoglobin A amino acid sequence by single amino acid substitutions at the sixth position of the β chain:

Hemoglobin	Amino-acid Substitution	Nucleotide Change
A → S	Glu → Val	GAG → GUG
A → C	Glu → Lys	GAG → AAG

As shown above, either one of these amino acid substitutions can occur as a result of a single base change in the triplet codon corresponding to that

position in the β globin gene. Hence in normal hemoglobin A individuals, these point mutations in the β globin genes in erythroid stem cells will give rise to circulating red cells containing hemoglobin S or C in addition to hemoglobin A. To identify such cells, the hemoglobin S antibody was conjugated with FITC and incubated with red cells fixed on slides. The preparations were then manually examined under a fluorescence microscope for the presence of antibody-labeled cells. Long and laborious counting effort revealed their presence at a frequency of about one labeled cell in 10^7 unlabeled cells.[5] While these results were encouraging, this assay method was not practical since a single measurement was so time consuming (about one man-month per sample) and it was not possible to demonstrate biochemically that the labeled cells did, in fact, contain hemoglobin S.

It was at this point we established a collaboration with Dr. Stamatoyannapoulos to explore the potential application of flow sorter technology to this problem. Two technical issues needed to be addressed; (1) could a hemoglobin antibody staining method be developed for red cells in suspension and (2) could the sorter process such samples rapidly enough to allow examination of a statistically significant number of cells in a reasonable time. To permit antibody labeling of hemoglobin, a method needed to be devised which would maintain the integrity of individual erythrocytes, fix intracellular hemoglobin <u>in situ</u> and then permit access to antibody. Such a procedure was suggested by the work of Wang and Richards,[6] in which membrane permerable cross-linking reagents were used to study the topography of red cell membrane proteins. They showed that, in intact red cells, these reagents cross-linked intracellular hemoglobin to itself and to many of the peripheral and intrinsic membrane proteins (Bands 1,2,3,4.1,4.2,5,6 and 7). We successfully adapted this procedure to cross-link cells and produce ghosts

which retain about 10^6 hemoglobin molecules covalently bound to the cell membrane, remain permeable to antibody and retain the native antigenicity of hemoglobin A, S and C.[7]

To test the specificity of antibody labeling, artificial mixtures of variant and normal cells were processed together and examined. Fluorescence microscope observation revealed the approximate number of expected fluorescent ghosts with the normal hemoglobin-containing ghosts nearly invisible. These ghost suspensions were then analyzed quantitatively on the FACS cell sorter. By integrating the number of fluorescence signals of intensity characteristic of antibody-labeled hemoglobin AS or AC ghosts and knowing the total number of ghosts processed by the sorter, the frequency of positive ghosts could be calculated and compared to the known frequency in the prepared mixture. As shown in Figure 1, artificial mixtures as dilute as one variant in $\sim 10^5$ normal cells could be accurately reconstructed before fluorescent background noise obscured the rare labeled ghosts.

Next, the issue of sorter processing speed was examined. Assuming a background frequency of one anti-hemoglobin S positive-cell per 10^7 negative cells to be correct, one would need to analyze at least 10^9 total cells per sample. To do this efficiently requires a sorter throughput rate of $\sim 10^6$ per sec, a rate that is about 300 times higher than is normally used. Such high flow rates require a sample density of about 5×10^8 cells/ml resulting in about 20 cells being in the laser beam simultaneously. Using cross-linked red cell ghosts, we wished to determine if rare fluorescent ghosts could be reliably detected under such conditions. Suspensions of fluorescent ghosts in the absence and the presence of dense suspensions of unlabeled ghosts were analyzed. As illustrated in Figure 2, such rare ghosts

could be detected with no loss of resolution under flow conditions resulting in an overall ghost processing rate of greater than 10^6 per sec.

Since unavoidable fluorescent background noise prevented a direct machine count of the background frequency of rare antibody-labeled ghosts from bloods of normal individuals, we designed a two-step method in which the sorter served as a powerful enrichment device. This procedure first involves sorting all objects of fluorescence intensities characteristic of antibody-labeled ghosts then immobiliziang the sorted sample on microscope slides. The labeled ghosts, which can be discerned from fluorescent debris by their characteristic shape and membrane-associated fluorescence, were then counted manually using a fluorescence microscope equipped with a computer controlled scanning stage. Typically, a sorted sample was enriched in fluorescent to non-fluorescent ghosts approximately 3000-fold over the initial ghost suspension and could be quantitatively scanned under the microscope in about six hours. Table I lists our initial results using this two-step method together with previously reported slide-based results obtained by Dr. Stamatoyannopoulos.[5] The Table also includes our results with the anti-hemoglobin C antibody. The frequencies of anti-hemoglobin S labeled cells using the two techniques are consistent and the S and C frequencies comparable. In spite of this initial success, this approach is compromised in several respects. The signals from the rare, dimly fluorescent ghosts cannot be separated from the fluorescent background artifacts, thus the slide preparations are contaminated and difficult to analyze. The ghosts themselves are very fragile structures which we could not immobilize without significant and variable losses. Thus are results are only semi-quantitative; a large part of the order of magnitude variation of the reported variant cell frequencies may be attributable to

these technical problems. We believe a more quantitative procedure is required in order to detect an increase in the number of variant cells with subtle environmental mutagen exposures. Also, the ghosts contain only about 1% residual hemoglobin which makes biochemical verification difficult.

"HARD-CELL" LABELING APPROACH

Because of the technical problems encountered with the ghost approach, we are exploring another red cell preparation procedure. A potentially useful strategy was suggested by the work of Aragon et al.,[8] in which red cells could be made permeable to substrates of intracellular enzymes without concominant loss of the proteins themselves. This was accomplished by heavy cross-linking with the same membrane permeable reagent, dimethyl suberimidate, used in the ghost procedure. The resulting "hard cells", resistant to hypotonic lysis, are then permeabilized by organic solvent/ detergent treatment. We have added a third step of protease digestion in order to remove the outer surface of residual membrane proteins to expose more of the immobilized intracellular hemoglobin. These "hard cells" possess a number of useful properties. They are mechanically very stable and do not lose significant amounts of hemoglobin. This physical stability will permit serial sorting of samples. By re-sorting the initial sorted sample, we expect to obtain significantly increased enrichments of labeled cells. In model experiments, using artificial mixtures of fluorescent and non-fluorescent "hard cells", approximately 90% recovery efficiencies were obtained in two-step serial sorts. Like the ghost suspensions, these cells can be processed at throughput rates exceeding 10^6 per sec. In addition, these cells can be easily and quantitatively immobilized on microscope slides.

Secondly, exterior presentation of the hemoglobin should improve the efficiency and specificity of antibody labeling thus lowering the frequency of fluorescent artifacts. Extended incubation time under less than optimal solubility conditions for IgG are necessary for immunologic labeling of ghosts. The resulting micro-precipitates of fluoresceinated antibody contribute significantly to the frequency of false-positive signals. With the "hard cell" approach, staining conditions can be modified for optimal stability of the antibody. Lastly, since essentially the full cellular hemoglobin content is retained in these "hard cells", almost 100-fold more protein per cell can be obtained for biochemical analysis. By using a reversible cross-linking analog of dimethyl suberimidate, 3,3' dithiobispropionimidate,[6] and ultra-thin gel[9] or single cell electrophoretic techniques,[10] it appears feasible to directly characterize the hemoglobin content of individual antibody labeled cells.

We are presently refining this technique for immunologic labeling; an initial result demonstrating specificity of FITC-anti-hemoglobin S binding to AS "hard cells" is presented in Figure 3. If accurate reconstructions of artificial mixstures can be obtained, we plan to adopt this procedure as the one of choice for continuing work.

FUTURE OF THE HEMOGLOBIN-BASED ASSAY

Continued progress on the development of this assay approach is dependent on three factors: (1) success of the "hard cell" preparative technique, (2), continued development of automated cytometric analysis methods and (3), availability of highly specific antibodies to hemoglobin variants. At LLNL

there are two ongoing machine development projects relevant to this work. The first is the construction of a high-speed sorter. This device operates at about 20 times the sample stream velocity and droplet formation rate of conventional cell sorters thus allowing an order of magnitude increase in processing speed. Secondly, a slit-laser illuminated microscope system has been constructed. This device has been used in the manual mode for examining microscope slides containing the sorted ghost samples but is also be capable of computer-controlled slide scanning and automated detection of labeled cells. We are also presently addressing the third point above with the development of mouse monoclonal antibodies to a variety of mutant hemoglobins. While whole animal methods have succeeded in producing monospecific antibodies to 11 additional single amino acid-substituted hemoglobins,[11] the development of the hybridoma technique offers the most promise for obtaining the desired reagents. This approach has already been successfully applied as mouse monoclonal antibodies to myoglobin and to hemoglobin have been reported.[12,13] In no other context can the inherent advantages of high purity, exquisite specificity and reliable antibody production of hybridoma-derived antibodies be better exploited than in this system. We have made a comprehensive survey of all known human hemoglobin single amino acid substitution variants and evaluated each for its applicability to this work. The criteria for inclusion were:

- the hemoglobin and/or variant cells be available in useful quantities
- the variant can result from a single base change in the hemoglobin A gene
- the variant is a stable hemoglobin, so mutant cells will not be selected against in vivo

- the substituted amino acid be recognizable by the mouse immune system
- a variety of base changes, particularly those leading to electrophoretic variants, be represented
- both α and β globin variants be represented

As shown in Table II, application of these criteria results in a list of 21 candidate single amino acid substituted hemoglobins in addition to S and C. We have begun initial immunizations using ten of these variants. Mice immunized with these variants, should respond with a family of monoclonal antibodies, the great majority of which will recognize regions of the hemoglobin molecule common to both the normal and variant forms. However, by screening positive clones against both the variants and hemoglobin A, it will be possible to identify those binding specifically to an altered protein. Ultimately, if such an approach is successful, a library of monoclonal antibodies each specific for a different and defined hemoglobin variant, can be generated. A battery of such antibodies could then be used together to detect a variety of variant hemoglobin-containing cells, thus improving the technical ease of the assay (since the frequency of antibody-labeled cells will be higher) and its generalizability, since damage at many sites in both the α and β globin genes, involving both DNA base transitions and transversions, could then be detected.

In addition to the single amino acid substituted-variants, there exist other variant hemoglobins produced by different mutational mechanisms. As shown in Figure 4, two such hemoglobins, Cranston and Tak, are characterized by elongated β globin chains as a result of nucleotide insertions having occured in the vicinity of the carboxyl terminal region of the β globin gene.[14,15] These are stable variants and make up 30-35% of the total

hemoglobin produced in heterozygous cells.[16,17] The 11 amino acid carboxyl-terminal peptide common to these variants is immunologically detectable since specific anti-hemoglobin Cranston has been produced in horses.[18] As discussed by Stamatoyannopoulos et al.,[18] this same extended β chain sequence will be produced whenever there is a deletion of one (or 3n+1) nucleotide or an insertion of two (or 3n+2) nucleotides in the β globin gene provided that such frameshifts do not lead to an unstable globin chain or create an accidental termination codon within the β globin gene. A mouse monoclonal antibody specific for any part of the extra peptide will thus detect a family of β globin gene frameshift mutations. This monoclonal antibody will be particularly valuable since it will detect a family of variant red cells distinct from the population of rarer variant cells arising from point mutations.

THE GLYCOPHORIN A SYSTEM

In parallel with the variant hemoglobin-based assay we are developing a second independent system based on the biochemically well-studied protein glycophorin A.[19] Glycophorin A is a glycosylated red cell membrane protein present at about 5-10 x 10^5 copies per cell.[20] Its 131 amino acid residues span the membrane with the amino-terminal portion presented on the red cell surface. The utility of this protein as a basis for a somatic cell mutation marker was suggested by the work of Furthmayer[21] which showed that this protein was responsible for the M and N blood group determinants and that these determinants were defined by a polymorphism in the

amino acid sequence of the protein coded for by a pair of co-dominantly expressed alleles. The polymorphic sequence at the amino-terminus is shown below:

Glycophorin A(M) Ser-Ser(*)-Thr(*)-Thr(*)-Gly-Val-...

Glycophorin A(N) Leu-Ser(*)-Thr(*)-Thr(*)-Glu-Val-...

(*) indicates a glycosylated amino acid

Except for the amino acid substitutions at positions one and five of the sequence, the two proteins are identical, both in amino acid sequence and sites and structures of glycosylation. Individuals homozygous for the M or N allele synthesize only the A(M) or A(N) sequence respectively, while heterozygotes present equal numbers of the two proteins on their erythrocytes.[21]

Two assays can be developed for the glycophorin A system. The first we call our glycophorin A "gene expression loss" or "null mutation" assay. In this approach, we wish to detect rare erythrocytes in the blood of glycophorin A heterozygotes which fail to express one or the other of the two allelic forms of the protein. Such an approach has been described for an in vitro system using human cells heterozygous for the multi-allelic HLA determinants.[22,23] Using immunologic selection, these researchers have demonstrated that lymphoid cells can lose expression of one or more polymorphic HLA cell surface antigens as a result of spontaneous mutation. Exposure to radiation or chemical mutagens increases the background mutation rate by greater than two orders of magnitude. Analysis of these variants showed the majority to be single HLA gene mutants.

The glycophorin A "gene expression loss" approach has several inherent practical and biological advantages over the the hemoglobin-based system. First, this antigen is presented on the surface of the red cell and is firmly anchored in the membrane; thus, cell preparation and antibody labeling procedures are simple and straightforward. Second, since the mutant phenotype detected can result from a variety of mutational lesions, i.e., single nucleotide changes and frameshifts occuring either in the glycophorin A structural gene or its control elements, the frequency of variant cells should be much higher (perhaps 100-1000 times the frequency seen for a single amino acid substitution at a single site). Hence such cells should be easier to detect and the frequency of such cells, representing the sum of all of these mutational mechanisms, may more accurately reflect the integrated genetic damage in that individual.

To detect the presence of these functionally hemizygous cells we are at present generating mouse monoclonal antibodies which differentiate the M and N forms of the protein. Mouse monoclonal antibodies recognizing glycophorin A have been produced by Edwards[24] and we have adopted a variation of his immunization protocol. First, mice were injected with a equal mixture of homozygous MM and NN red cells, then boosted with purifed glycophorin A(M) and A(N). The serum was then assayed for the presence of anti-red cell antibodies. The spleens from responding mice were then fused with SP2/0 mouse myeloma cells and anti-red cell producing clones were selected using a red cell enzyme-linked immunosorbant assay (ELISA). Positive clones were then assayed using homozygous MM and NN cells and those showing specificity for either cell type were selected, sub-cloned and expanded. Using this procedure, we have isolated four clones, two of which are specific for

glycophorin A(M), one specific for A(N) and one which recognizes a shared determinant. Purified A(M)- and A(N)-specific monoclonal antibodies will be labeled with green and red fluorophors, e.g., fluorescein isothiocyanate (FITC) and a derivative of rhodamine isothiocyanate (X-RITC) and incubated similtaneously with red cells from MN heterozygotes. Variant cells, defined by binding of only one of the antibodies and hence fluorescing only green or red, will be enumerated as shown in Figure 5 using the LLNL two-color dual-beam flow sorter.[25] The variant frequency will simply be the number of green- or red-only cells divided by the total number of cells processed (the sum of the signals in all three peaks). Because we expect the frequency of these variant cells to be as much as a 1000-fold higher than the frequency of single amino acid substitution-variant cells, direct sorter quantitation should be possible. Also adequate numbers of variant cells should be obtainable by sorting for biochemical analysis.

Since this assay is based on the detection of cells which fail to express a gene product it is critical to insure that the counted variant cells are true glycophorin A structural gene mutants. This is important since there are both genetic and non-genetic mechanisms which could cause the protein to fail to appear on the red cell membrane. For example, mutations outside the glycophorin A locus leading to loss of function of proteins necessary for processing, transporting, glycosylating or inserting glycophorin A into the membrane could produce apparent glycophorin A "null" cells. Non-genetic events include loss of membrane integrity or insufficient levels of substrate sugars for glycosylating enzymes due to metabolic anomalies. This assay is strongly protected against such false positive "phenocopies" since it requires antibody binding to one of the glycophorin A types. The proper cell surface

presentation of the glycophorin A product of the unaffected allele insures that the rest of the cell apparatus necessary for the expression of the protein is intact. Finally, we can be assured that the variant cells will not be selected against in vivo since erythrocytes from genetically homozygous glycophorin A "null" individuals, completely lacking expression of the protein, appear to exhibit normal viability.[26]

Our second glycophorin A-based assay corresponds exactly to the hemoglobin-based single amino acid substitution approach by taking advantage of the mutational basis underlying the A(M), A(N) polymorphism. Not unexpectedly, the sequence differences at positions one and five can arise as a result of single nucleotide changes in the glycophorin A gene:

	Amino-Acid Difference		Corresponding Triplet Codons	
	A(M)	A(N)	A(M)	A(N)
Position 1	Ser	Leu	TC(A or G)	TT(A or G)
Position 5	Gly	Glu	GGG	GAG

Thus in homozygous MM individuals there should be rare cells containing "N-like" glycophorin A with Leu at position one or, independently, Glu at position five. Likewise in the blood of homozygous NN people should be rare cells with "M-like" glycophorin A with amino acids Ser or Gly at positions one and five respectively. To detect such single substitutions our A(M)- and A(N)-specific monoclonal antibodies must recognize the amino acid differences at positions one and five independently, i.e., the antibody must not depend on

the presence of both differences for its binding specificity. It is most likely that our antibodies recognize the amino acid difference at position one. The amino-terminus of the molecule appears to be the immunodominant determinant recognized by animal anti-M and anti-N sera,[27] although some sera may be directed at position five.[28] We are presently testing the specificity of our clones with chemically modified glycophorin A to precisely define their target antigenic sites. Given the expected specificity, we will use these antibodies to measure the frequency of these singly amino acid substituted glycophorins in the blood of homozygous MM and NN individuals. These two assays of glycophorin A variant-cells will be particularly useful since it will be possible to compare the frequency of a single nucleotide change with the frequency of the loss of expression of the entire gene in the same structural locus.

SUMMARY

This paper has outlined our approaches for measuring somatic cell mutations in human erythrocytes using high speed sorter technology. These devices are capable of processing large numbers of cells with statistical precision and quantitatively sorting antibody labeled, presumptively variant, cells for subsequent analysis. All of our methods are based on immunologic detection of variant cells and hence depend on the availability of highly specific antibody reagents. Monoclonal antibodies are ideally suited for this purpose and are now in hand for the glycophorin A-based assays and under development in our laboratory and elsewhere for the hemoglobin-based assay. Armed with a battery of these hybridoma reagents it should be possible to

detect variant erythrocytes arising from an ensemble of mutations in the α-,β-globin and glycophorin A genes. Before practical application, these assays must be validated by measurement of reproducible background rates in "unexposed" control populations, dose response in mutagen-exposed individuals and direct biochemical verification of sorted variant cells.

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DISCLAIMER

FIGURE LEGENDS

Fig. 1 Reconstruction of artificial mixtures of hemoglobin AS and hemoglobin AA cells. AS and AA cells were mixed, ghosts prepared and the suspension incubated with FITC-anti-hemoglobin S. The mixtures were then analyzed on the cell sorter as described in the text. Similar results were obtained for AC/AA mixtures labeled with FITC-anti-hemoglobin C.[7]

Fig. 2 High-speed detection of fluorescent red cell ghosts in the presence of a high density of non-fluorescent red cell ghosts. Fluorescent red cell ghosts were first analyzed alone at a normal rate of approximately 10^3 per sec (top panel). The fluorescent ghosts were then mixed with a 1000-fold excess of unlabeled ghosts and the mixtures analyzed at the same rate (lower panel). Under these conditions the overall throughput rate of the unlabeled ghosts exceeded 10^6 per sec with no degradation in the histogram generated by the labeled ghosts.

Fig. 3 Specific binding of FITC-anti-hemoglobin S to heterozygous AS "hard cells". Hemoglobin AS and AA red cells were prepared as described in the text; 10^8 "hard cells" were then incubated for 30 min at room temperature with the indicated concentration of FITC-anti-hemoglobin S, washed three times in detergent buffer and analyzed on the flow sorter. The plotted intensities correspond to the peak modal channels of the resulting histograms.

Fig. 4 Amino acid and nucleotide sequences for normal βchain, (β Cranston) and (β Tak). These variant hemoglobins, characterized by 11 additional amino acids at the amino-terminus of the β globin chain, result from the insertion of two nucleotides (underlined) in the normal β globin gene. Any frameshift mutation which produces this same altered reading frame in an otherwise normal β globin gene will cause the synthesis of this same 11 amino acid peptide. Amino acid sequences are from Burn et al.,[14] and Flatz et al.,[15]. Nucleotide sequence is from Lawn et al.,[29].

Fig. 5 Dual beam sorter, two color fluorescent detection of glycophorin A "null" variant red cells. The figure depicts a hypothetical two-color fluorescence histogram of heterozygous glycophorin A red cells incubated with monoclonal FITC-anti-glycophorin A(M) and X-RITC-anti-glycophorin A(N). The green and red fluorescence will be detected independently with 488 nm argon laser excitation of FITC and 568 nm krypton laser excitation of X-RITC. The normal cells express both the M and N glycophorin A sequences; they will bind both antibodies and exhibit both green and red fluorescence. The variant cells, lacking the expression of one allele, will fluoresce only green or red.

Table I Frequencies of anti-hemoglobin S- and C-labeled red cells in the bloods of normal individuals. The LLNL results were obtained using the two step sorting-automated microscope scanning technique described in the text. The University of Washington results, using the manual slide-based technique, have been reported previously.[5] The numbers in parentheses indicate the number of individuals assayed.

Table II Candidate single amino-acid substituted hemoglobins for the production of mouse monoclonal antibodies. These variant hemoglobins, selected by the criteria outlined in the text, should stimulate mouse antibodies specific for the variant protein. Hybridomas, secreting such antibodies, will be selected and grown to serve as permanent sources of these reagents.

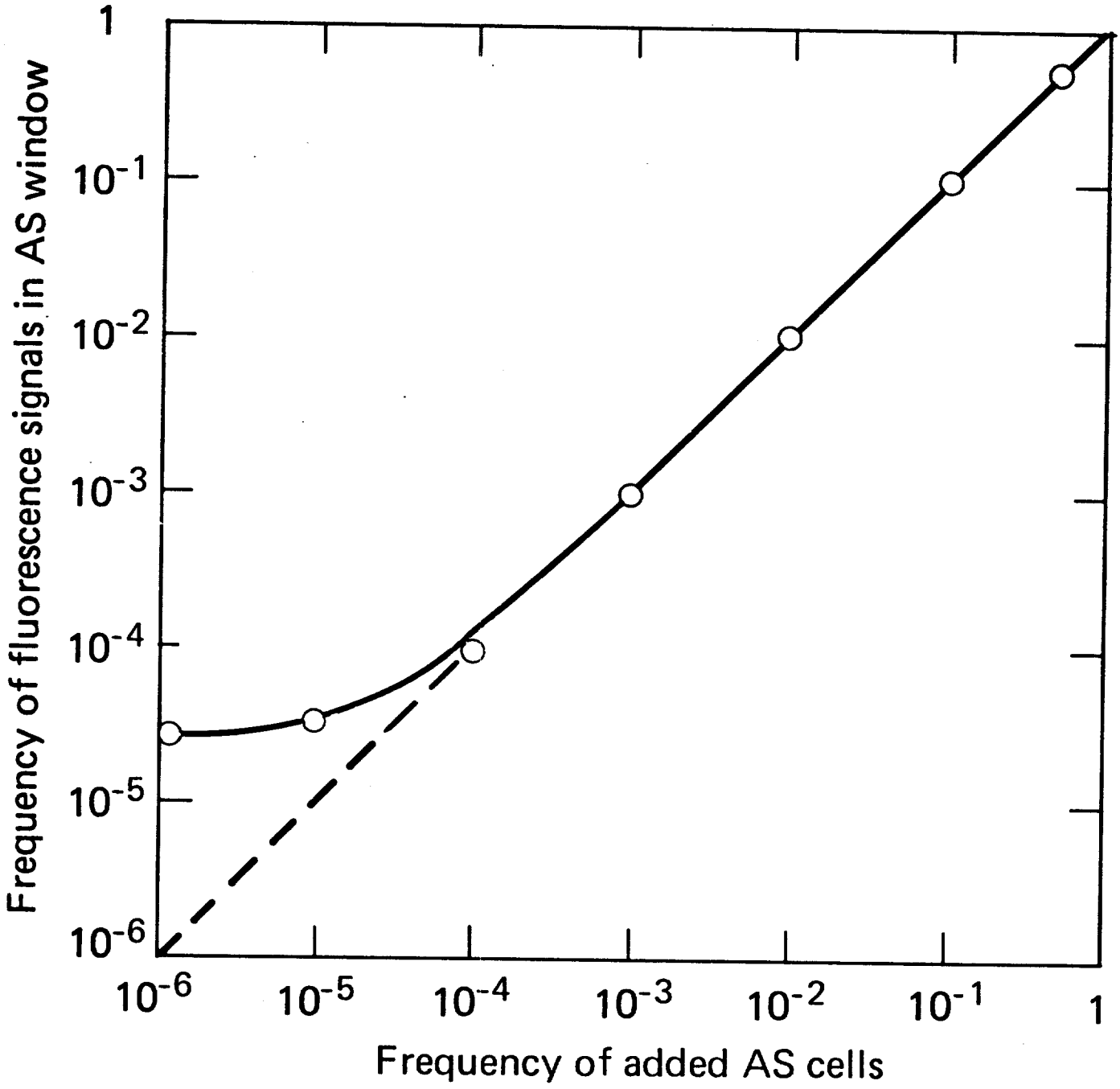

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Fig. 1

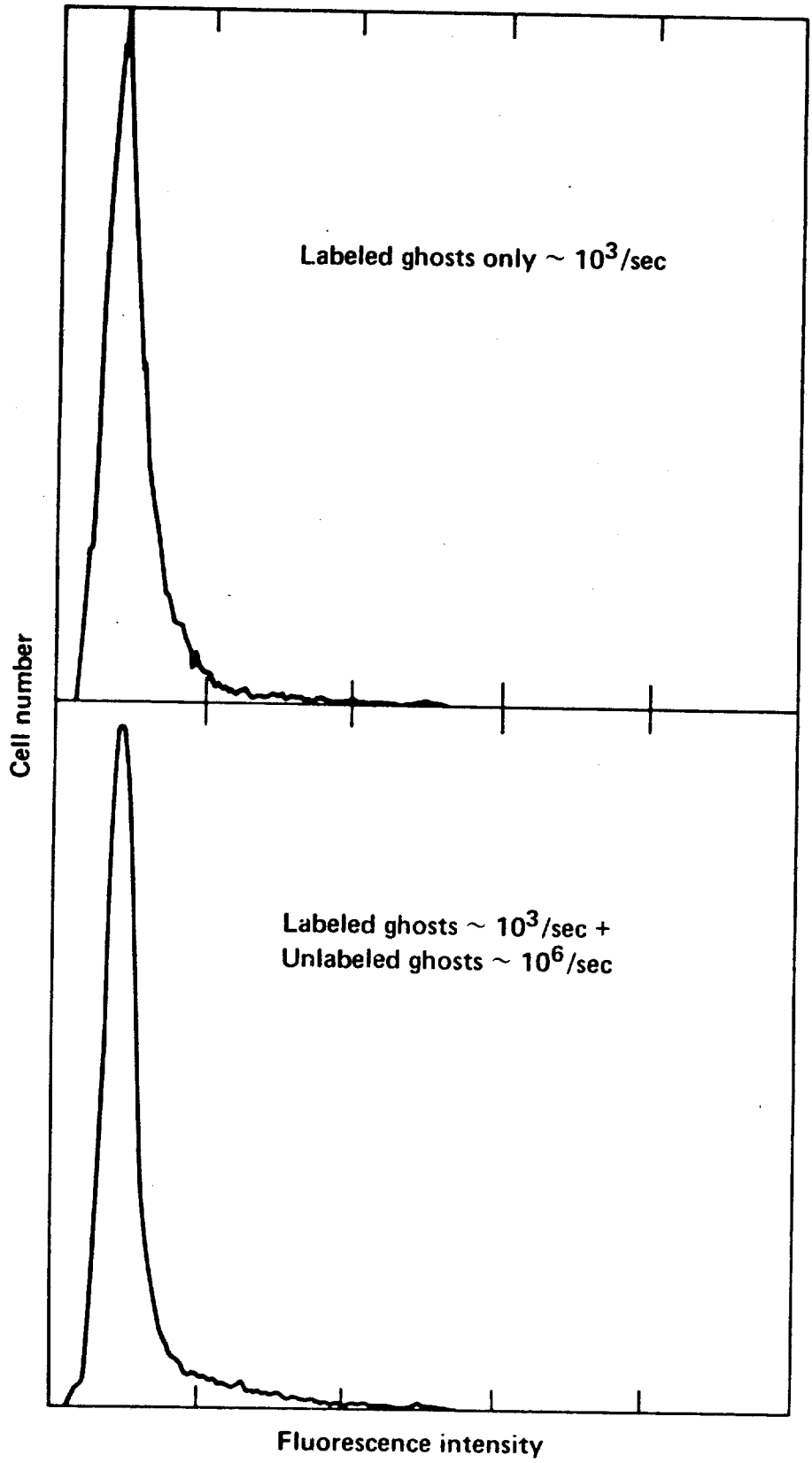

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Fig. 2

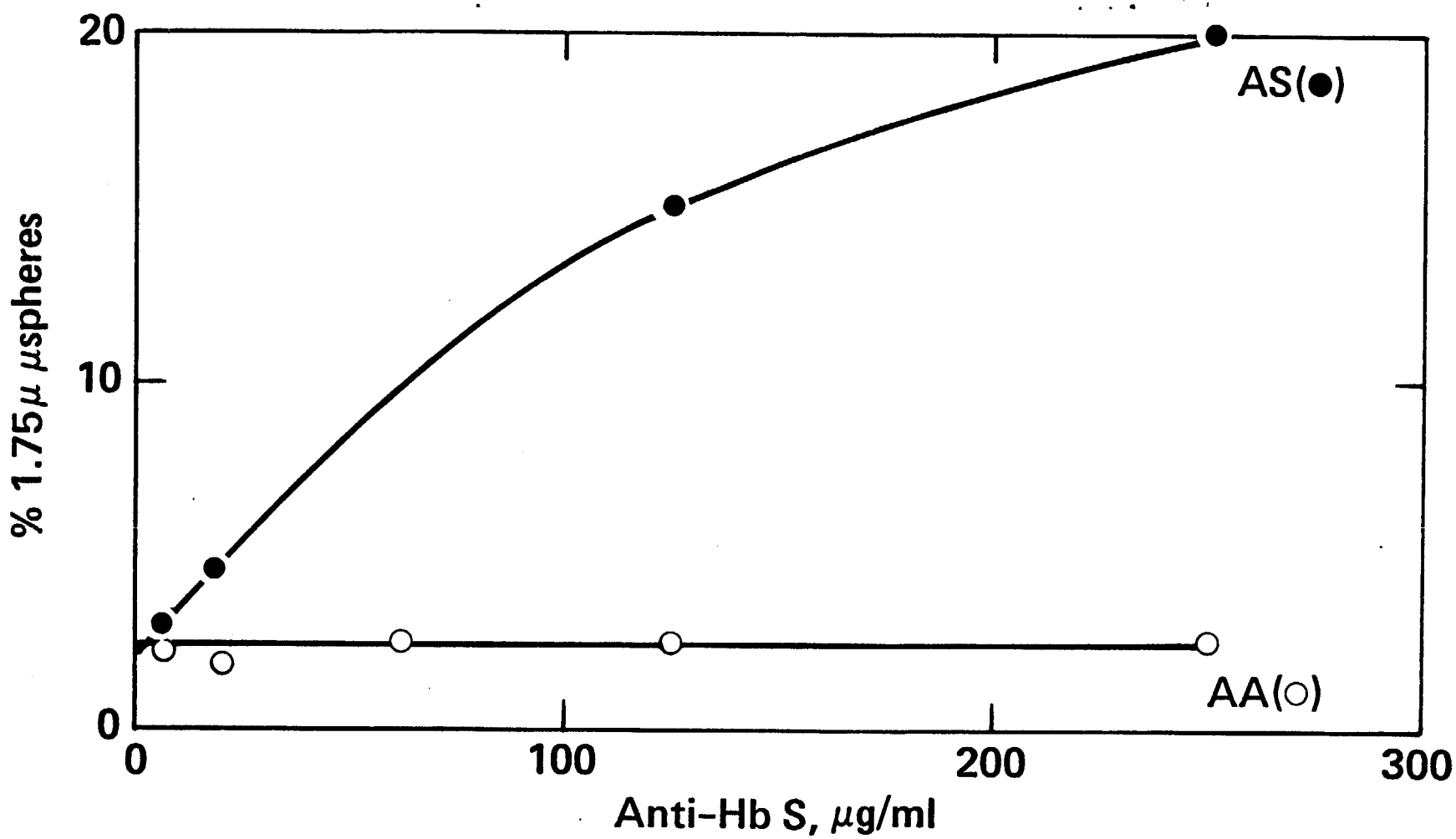

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Fig. 3

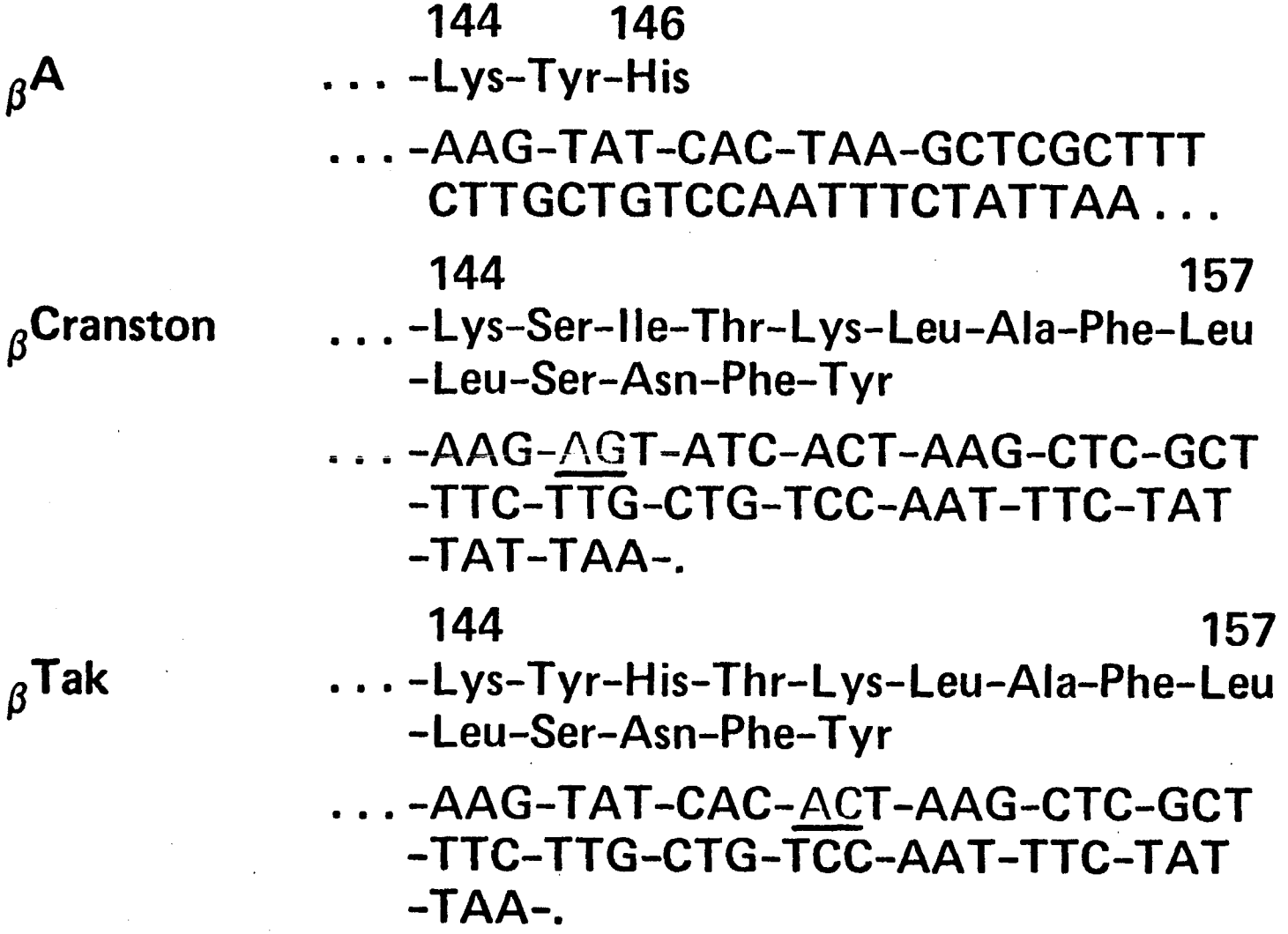

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Fig. 4

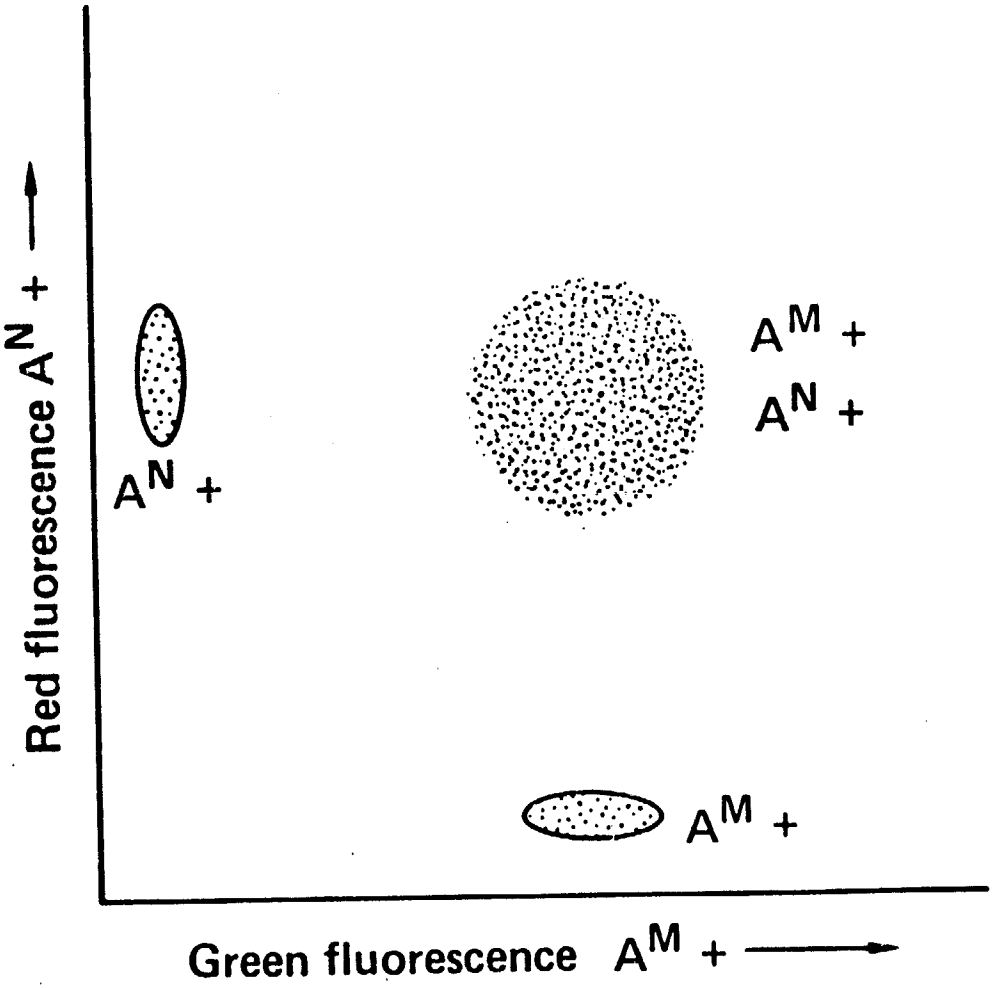

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Fig. 5

Table I

Hb S

(LLNL)	$1.1 \times 10^{-8} - 1.1 \times 10^{-7}$	(5)
(U. of Wash.)	$4 \times 10^{-8} - 3 \times 10^{-7}$	(15)

Hb C

(LLNL)	$6.7 \times 10^{-8} - 2.6 \times 10^{-7}$	(3)

Table II

Position	Hemoglobin Variant	Amino Acid Substitution	Base Change
α-Chain			
54	Mexico, J, J-Paris-II, Uppsala	Gln → Glu	C → G
	Shimonoseki, Hiroshima	Gln → Arg	A → G
56	Thailand	Lys → Thr	A → C
	Shaare, Zedek	Lys → Glu	A → G
57	L-Persian Gulf	Gly → Arg	G → A
	J-Norfolk, Kagoshima, Nishik-I, II, III	Gly → Asp	G → A
60	Zambia	Lys → Asn	G → T or C
	Dagestan	Lys → Glu	A → G
61	J-Buda	Lys → Asn	G → T or C
85	G-Norfolk	Asp → Asn	G → A
	Atago	Asp → Tyr	G → T
	Inkster	Asp → Val	A → T
90	J-Broussais, Tagawa-I	Lys → Asn	G → T or C
	Rajappen	Lys → Thr	A → C
120	J-Meerut, J-Birmingham	Ala → Glu	C → A
β-Chain			
20	Olympia	Val → Met	G → A
43	G-Galveston, G-Port Arthur, G-Texas	Glu → Ala	A → C
	Hoshida, Chaya	Glu → Gln	G → C
87	D-Ibadan	Thr → Lys	C → A
95	N-Baltimore, Hopkins-I, Jenkins, N-Memphis Kenwood	Lys → Glu	A → G
	Detroit	Lys → Asn	G → T or C